

# Neferine induces autophagy-dependent cell death in apoptosis-resistant cancers *via* ryanodine receptor and Ca<sup>2+</sup>-dependent mechanism

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**Running title:** Neferine induces autophagic cell death *via* RyR

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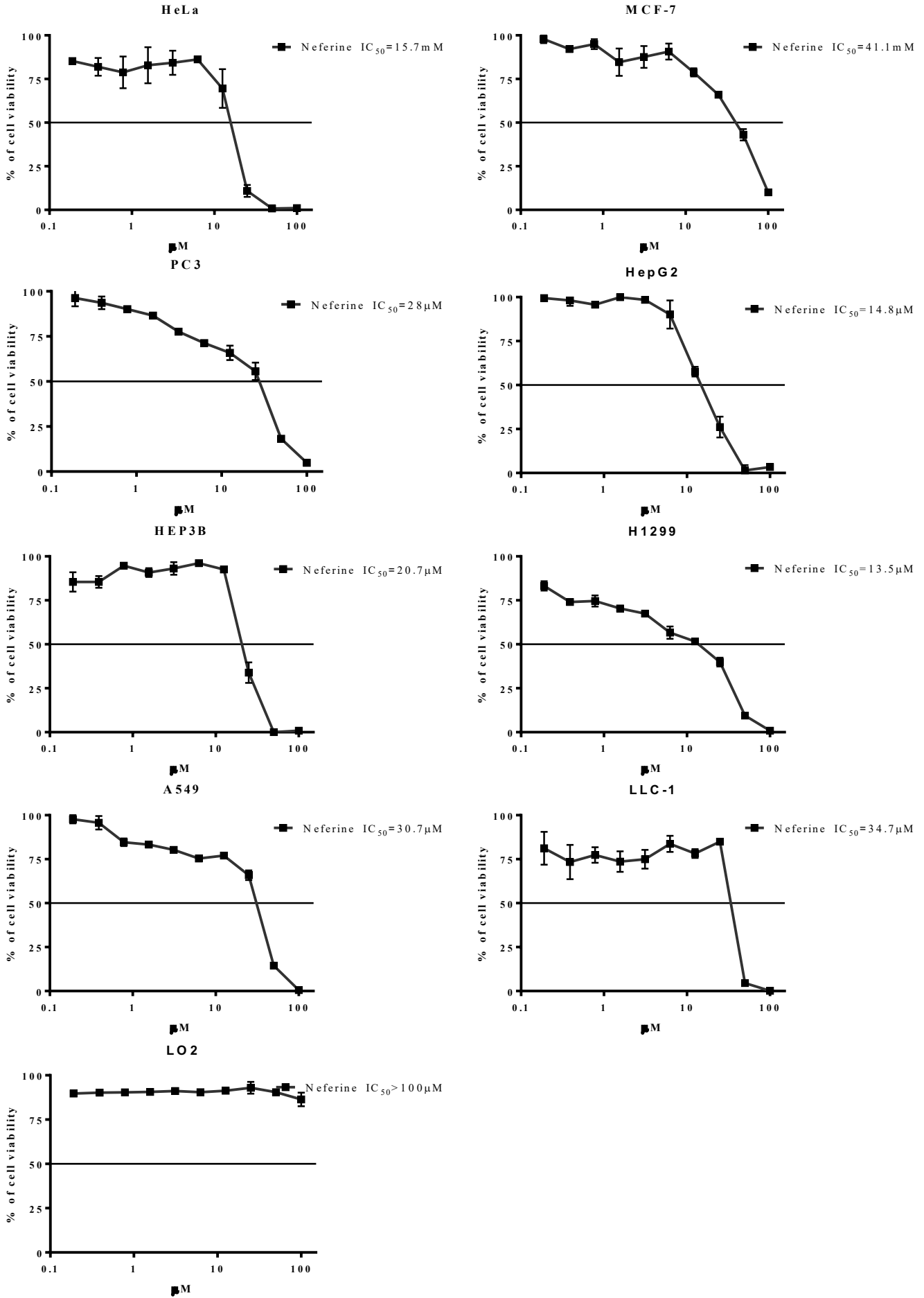
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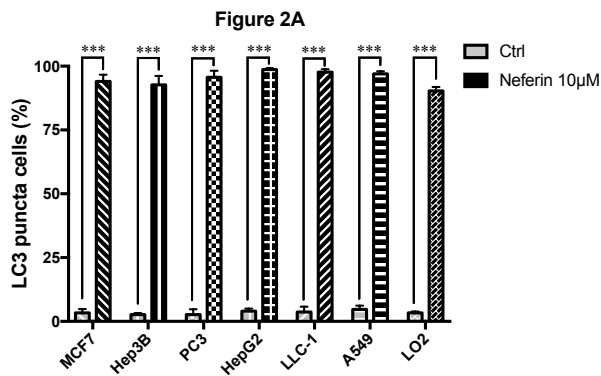
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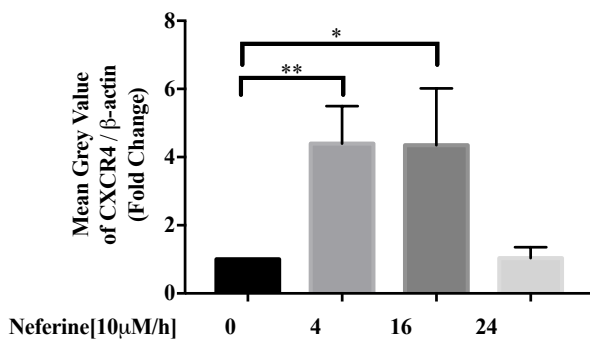
# Supplementary Figure (MTT graphs of cell lines in Figure 1B)



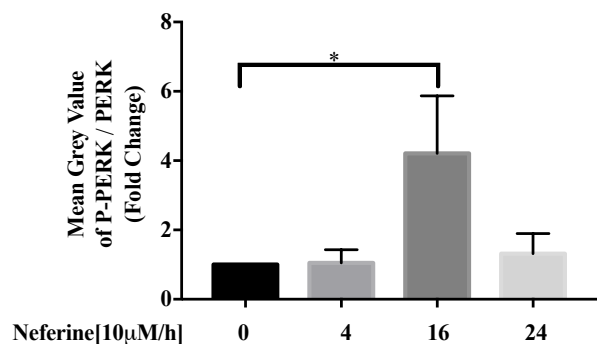
## Supplementary Figure (Quantification graphs of Figure 2A & 3B)



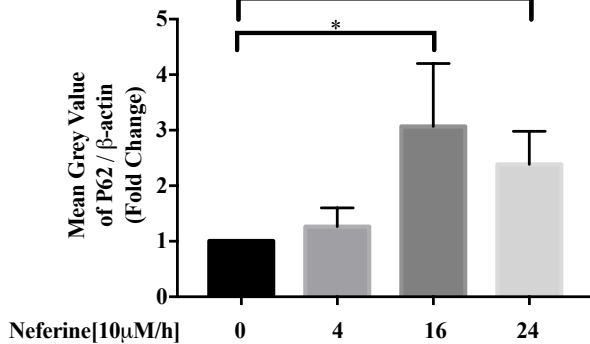
**Figure 3B CXCR4**



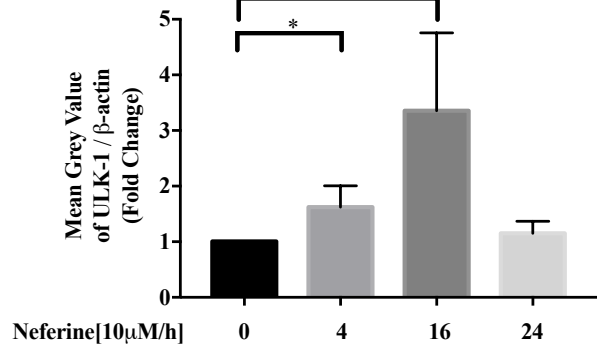
**Figure 3B P-PERK/PERK**



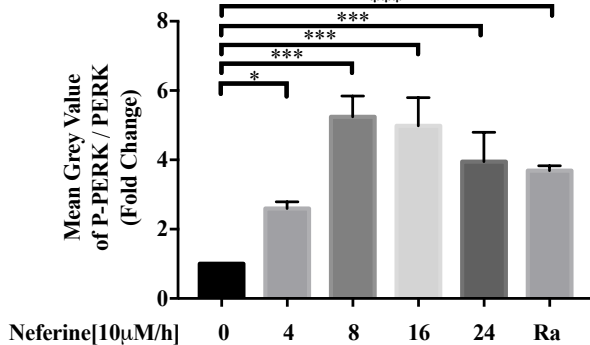
**Figure 3B P62**



**Figure 3B ULK-1**

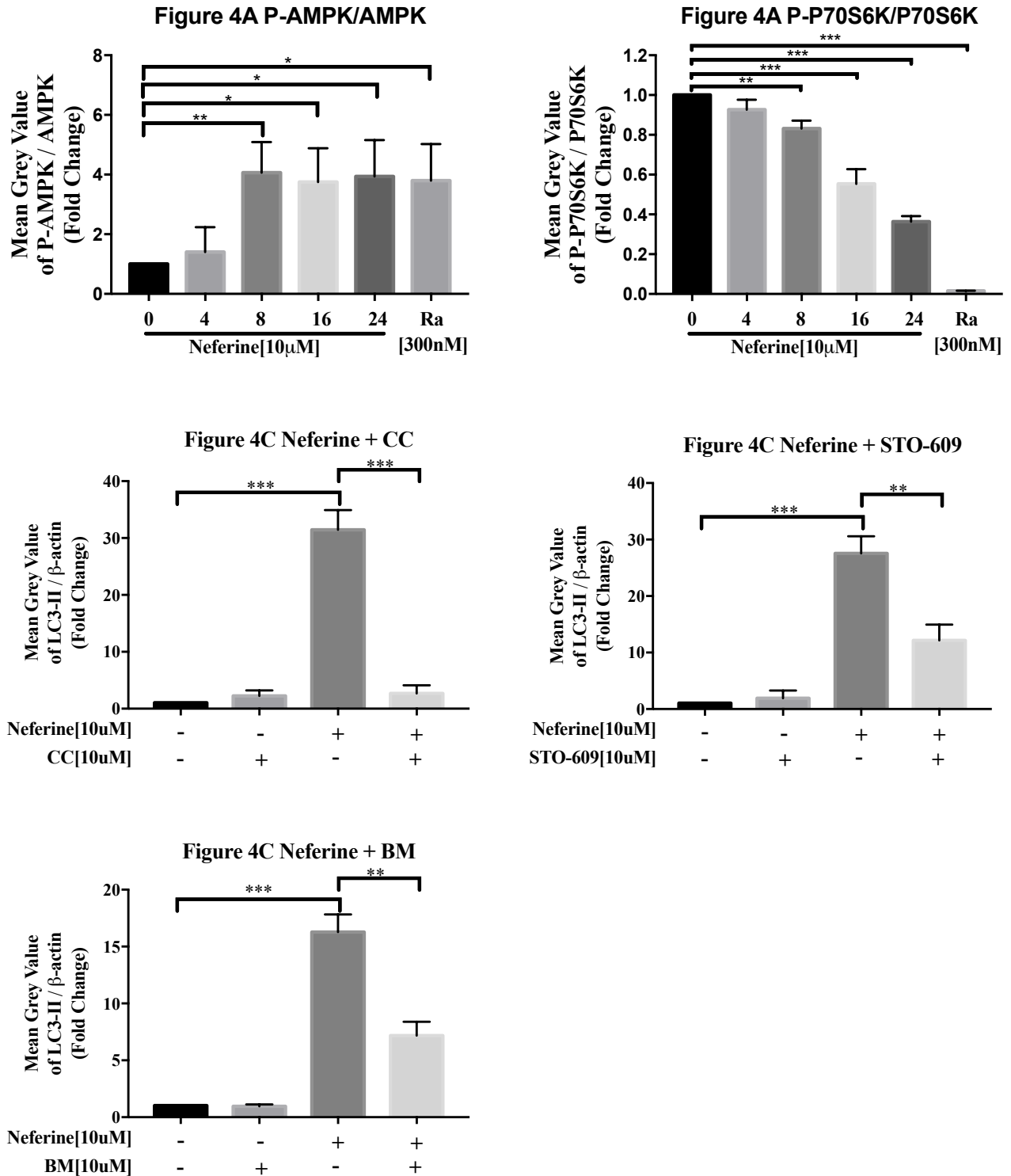


**Figure 3B P-eIF-2α/eIF-2α**

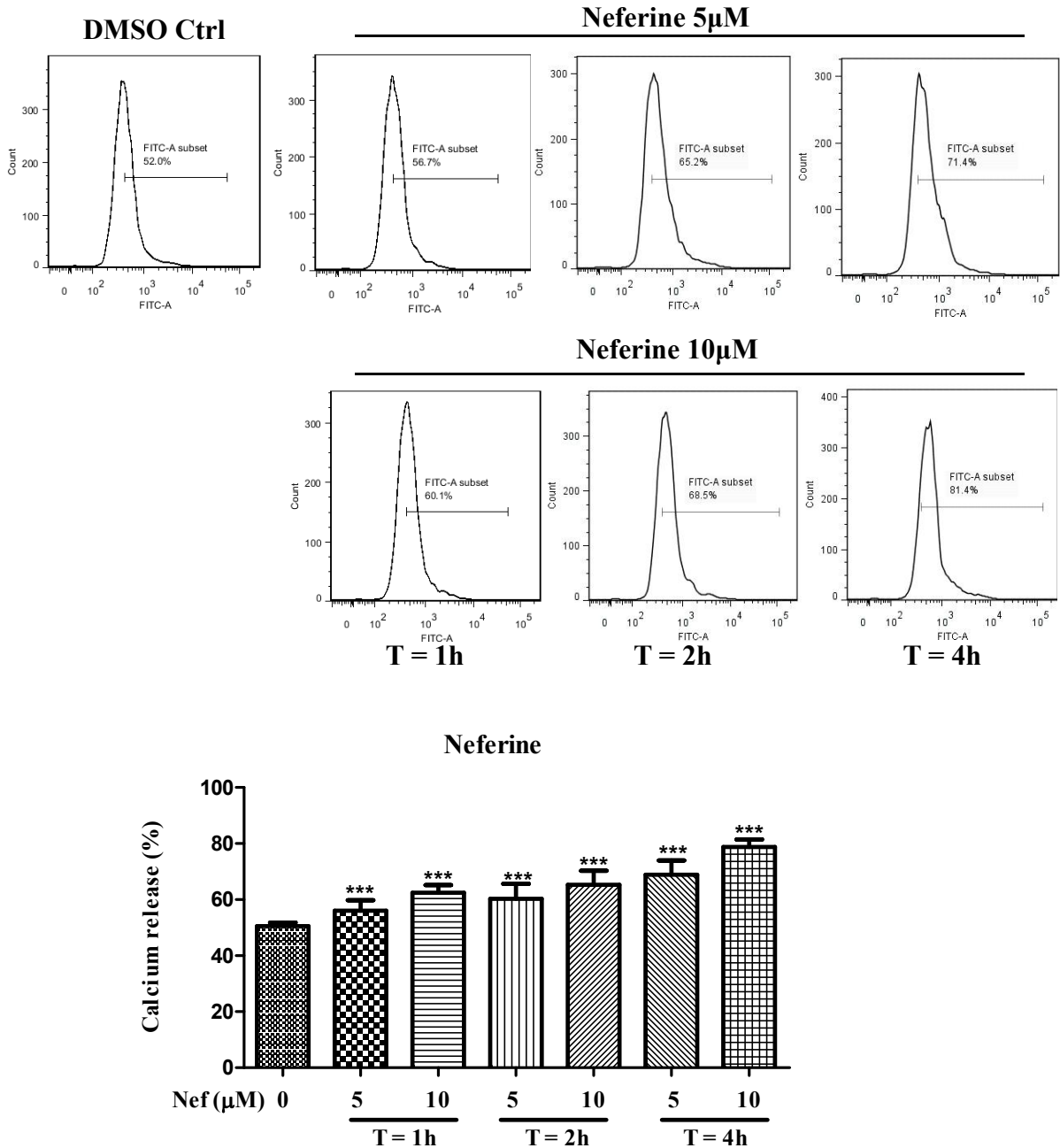


**Supplementary Figure S2.** The quantification of green endogenous LC3-II puncta formation (%) in Fig. 2A, compared to control group,  $***P \leq 0.001$ , *t test* analysis. And the quantification of mean grey value of western blot in Fig. 3B, compared to control group,  $*P < 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$ , one-way ANOVA analysis.

## Supplementary Figure (Quantification graphs of Figure 4A & 4C)

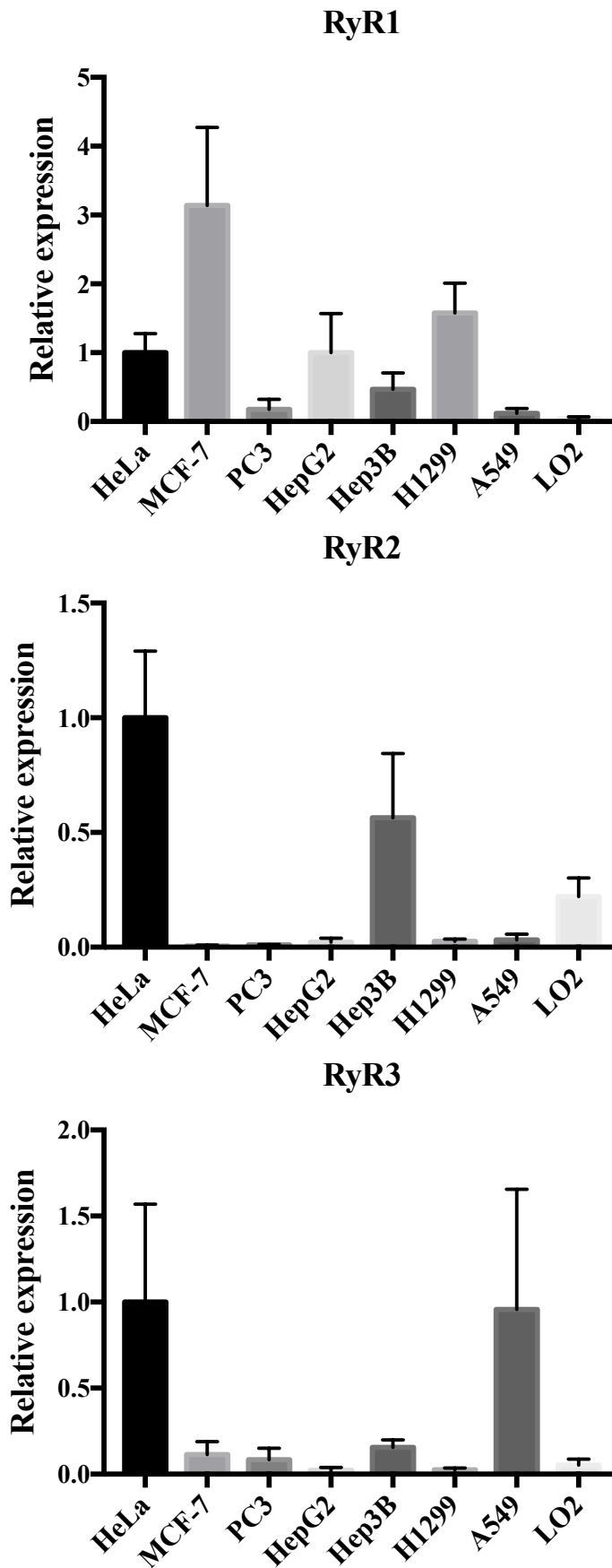


**Supplementary Figure S3.** The quantification of mean grey value of western blot in Fig. 4A, compared to control group, \* $P < 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , one-way ANOVA analysis. And the quantification of mean grey value of western blot in Fig. 4C, compared to neferine [10μM] group, \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , one-way ANOVA analysis.

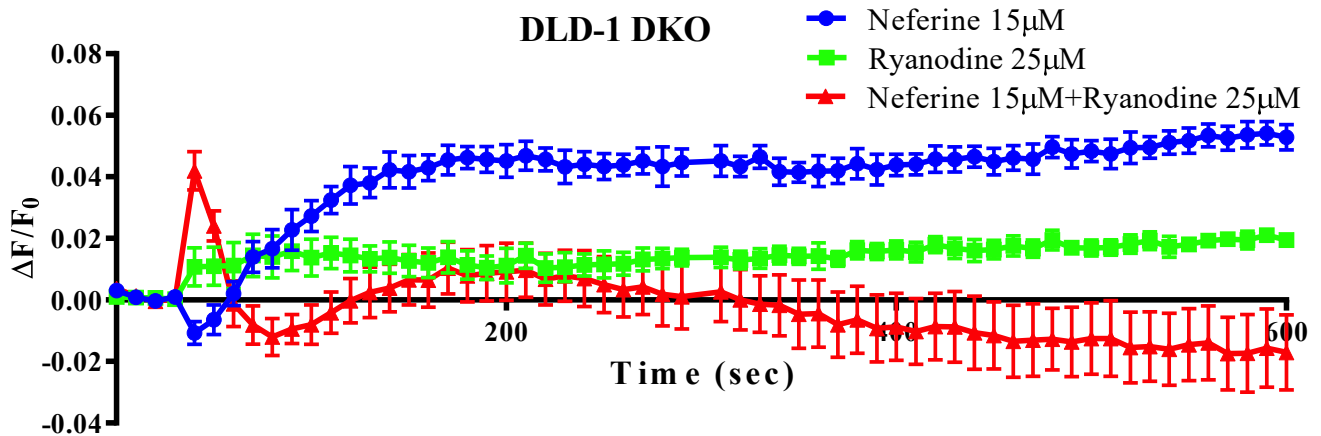


**Supplementary Figure S4.** Nef mobilized cytosolic calcium level in dose and time dependent manner. HeLa cells were treated with 5 or 10  $\mu$ M of Nef for indicated time. Intracellular  $\text{Ca}^{2+}$  concentrations were determined in terms of the fluorescent activity of Fluo-3 by FACS analysis. FACS charts shown are the representative of three independent experiments. Bar charts indicate the percentage of calcium release in cells; error bars, S.D. \*\*\*,  $P < 0.001$ .

## Supplementary Figure (RyR1/2/3 genes expression level in Figure 1B cell lines)



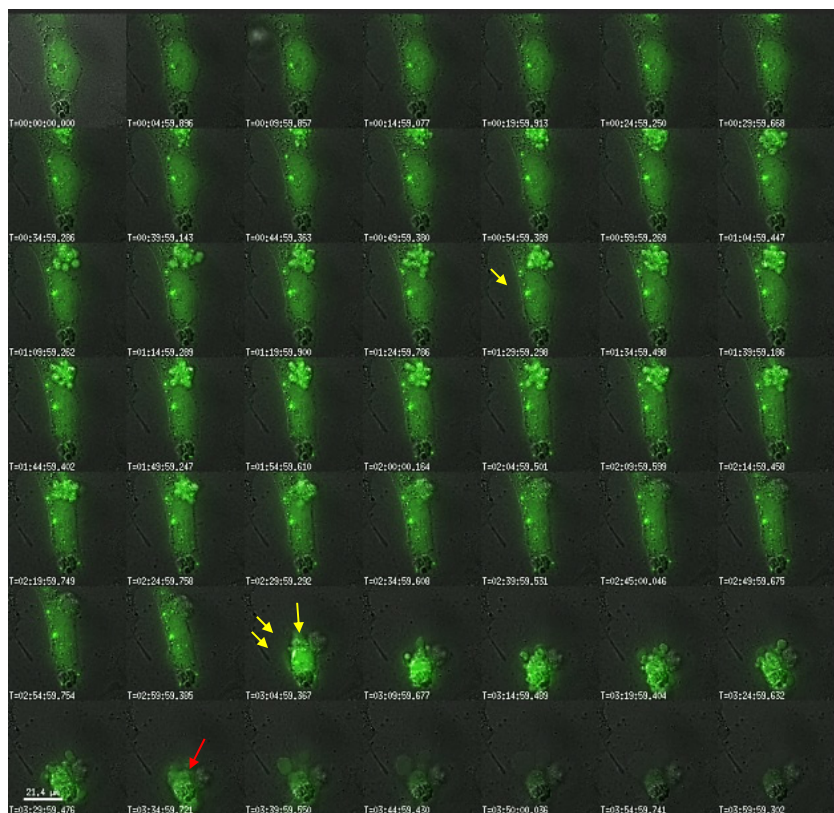
**Supplementary Figure S5.** The relative expression level (normalized to HeLa) of RyR 1/2/3 genes in 8 cell lines performed in Fig. 1B.



**Supplementary Figure S6.** Neferine-induced calcium dynamic change in DLD-1 Bax-Bak DKO colon cancer cells were abolished by addition of ryanodine receptor inhibitor (Ryanodine). DLD-1 DKO cancer cells stained with FLIPR Calcium 6 Assay Kit were treated with 15  $\mu$ M of neferine in the presence or absence of 25  $\mu$ M ryanodine, then immediately subjected to calcium dynamic measurement by FLIPR Tetra High-Throughput Cellular Screening System. Data from the chart represents mean values  $\pm$  S.D. of three independent experiments.

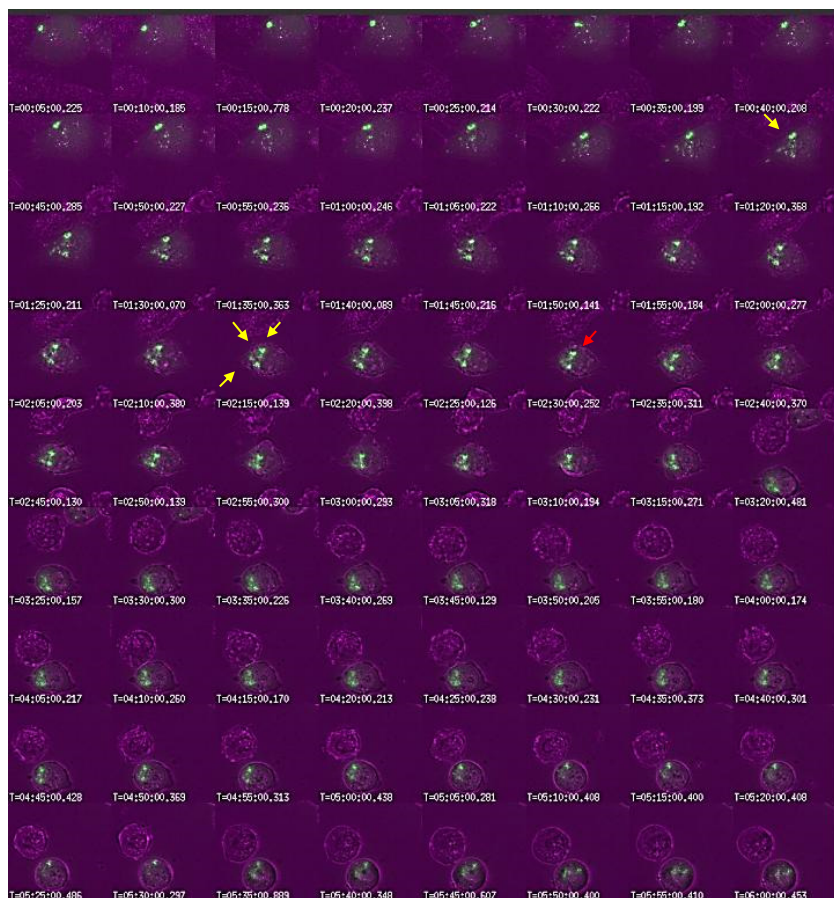
A

HeLa



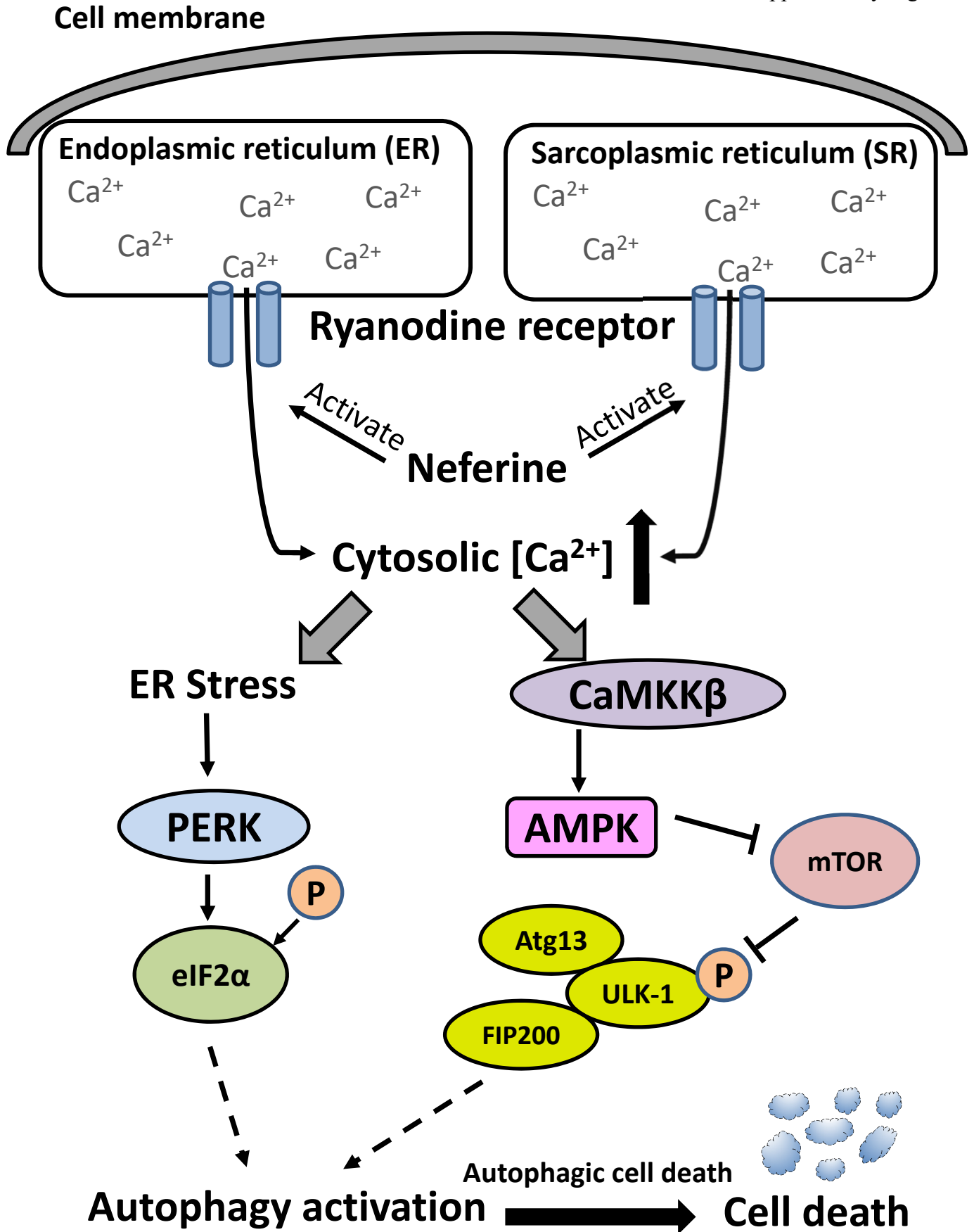
B

DLD1 Bax-Bak-DKO



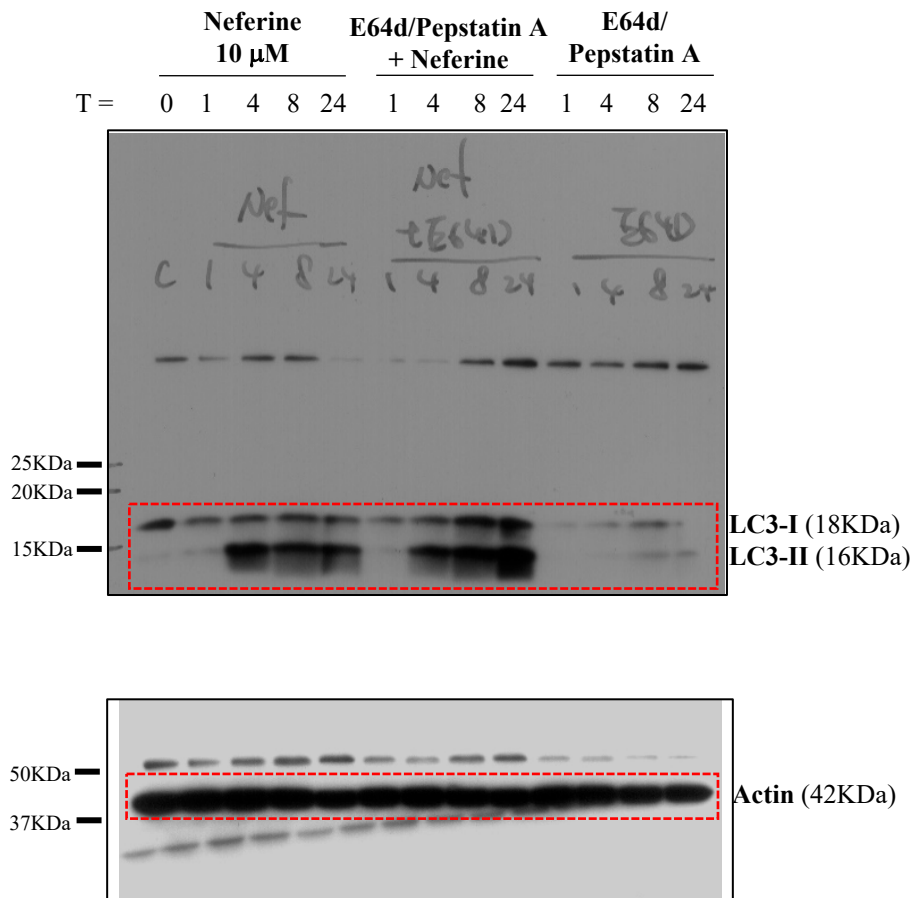
**Supplementary Figure S7.** Live-cell imaging of Nef-mediated autophagic cell death. EGFP-LC3 transfected HeLa cancer cells or apoptosis-resistant cells, DLD1 Bax-Bak DKO cancer cells were incubated at imaging chamber with 37°C supplied with 5% of CO<sub>2</sub>. After addition of 10  $\mu$ M of neferine, live-cell imaging was started to record the fluorescence and bright field images for 4 h with 10 min time-intervals. Arrows (yellow), autophagy induction with EGFP-LC3 accumulation (green); arrows (red), autophagic cell death.



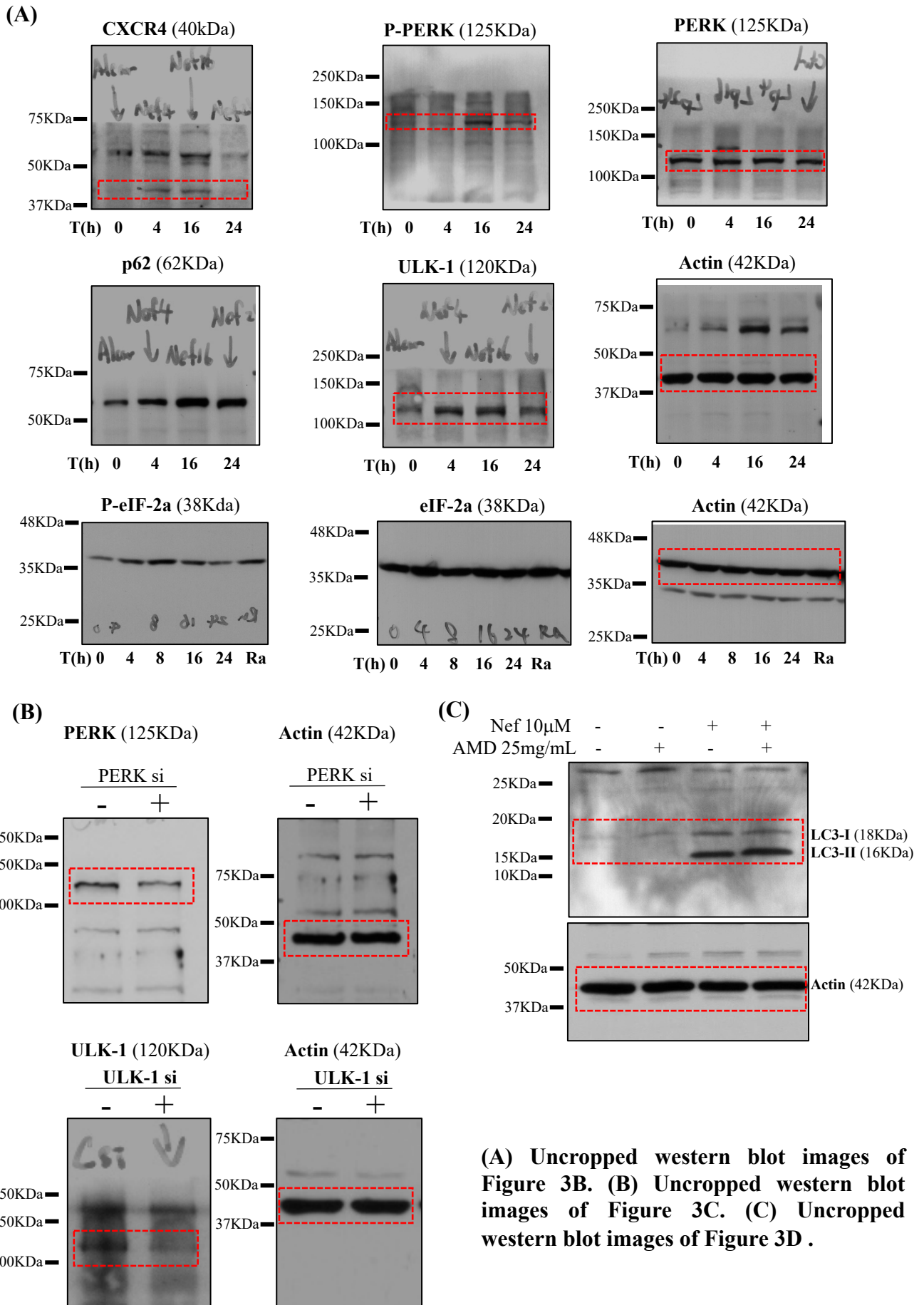


**Supplementary Figure S8.** Schematic diagram to illustrate the proposed molecular target and mechanisms of neferine in autophagy induction and its subsequent autophagic cell death.

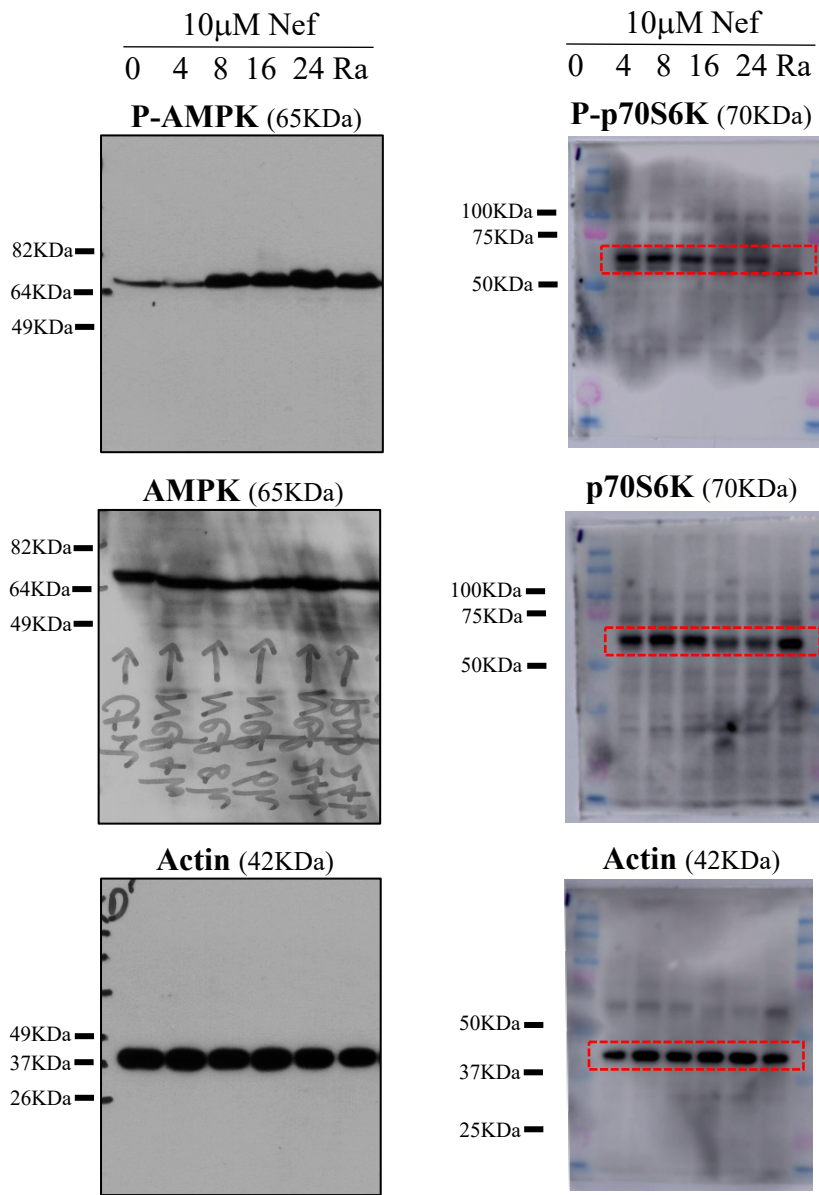
## Supplementary Figure (Uncropped western blot images)



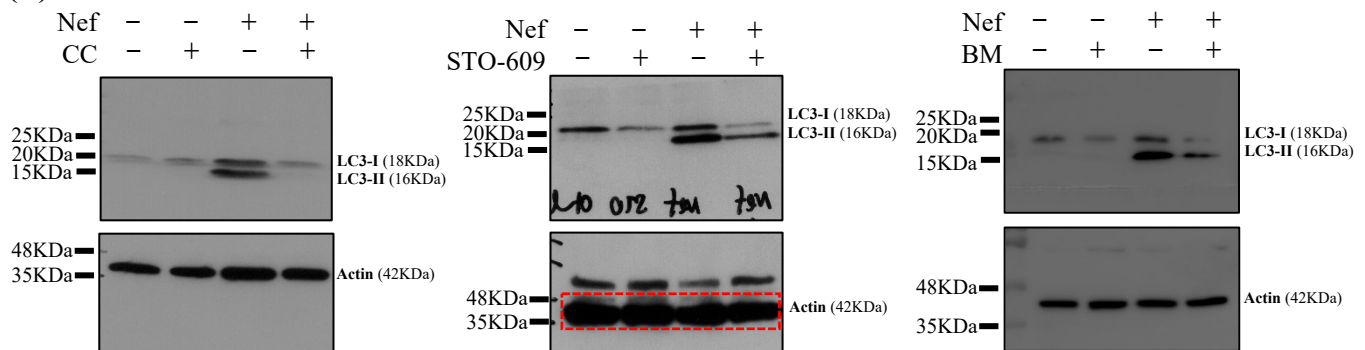
Uncropped western blot images of Figure 2C



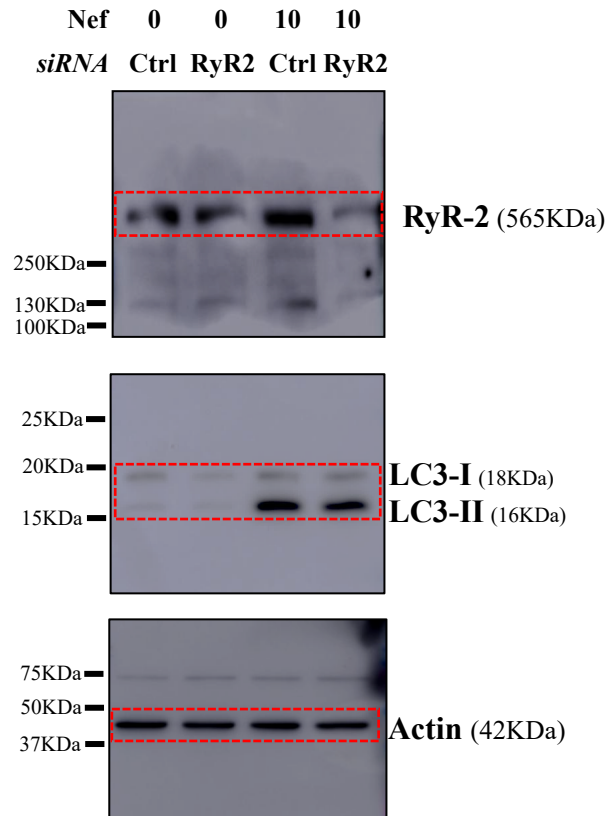
(A)



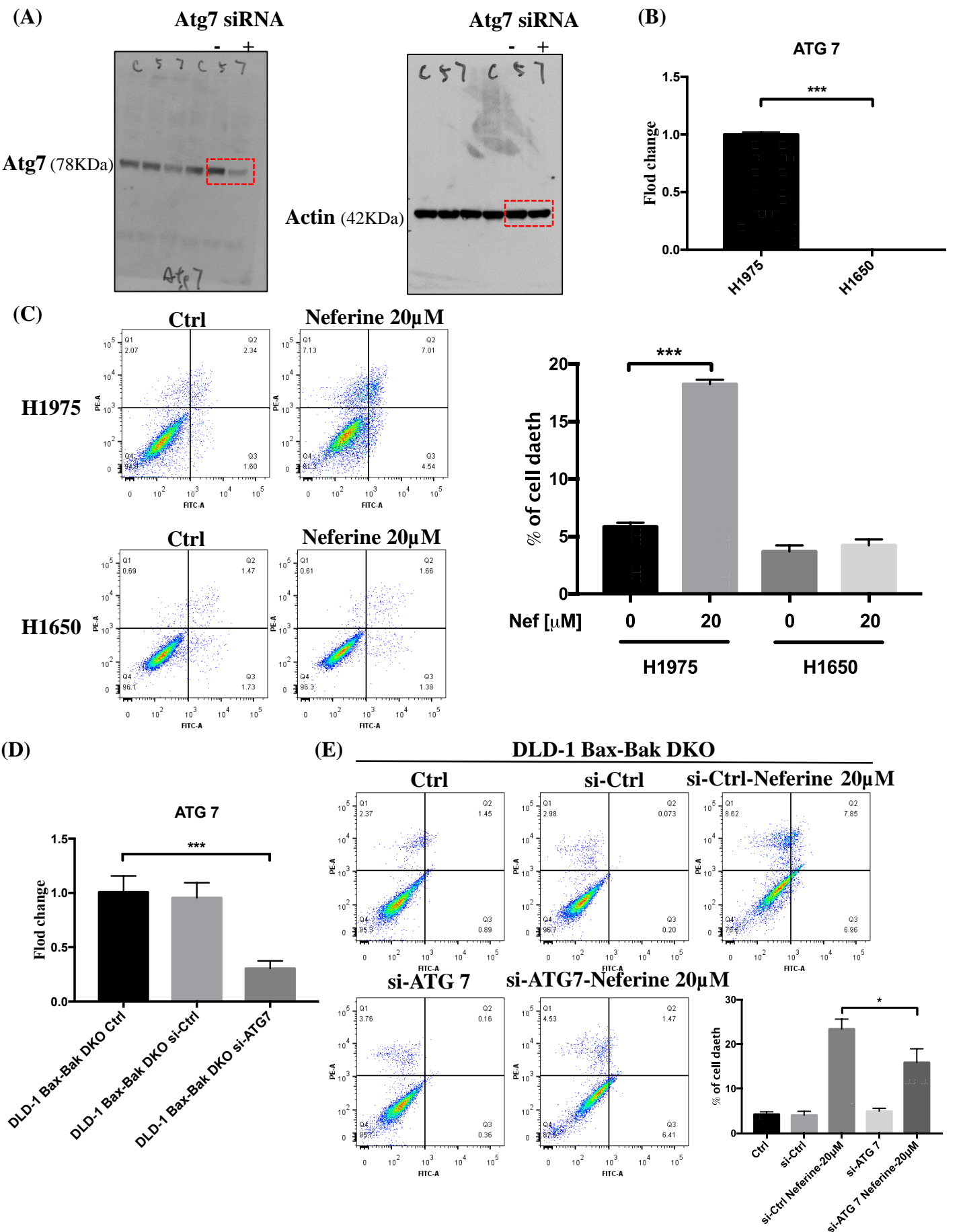
(B)



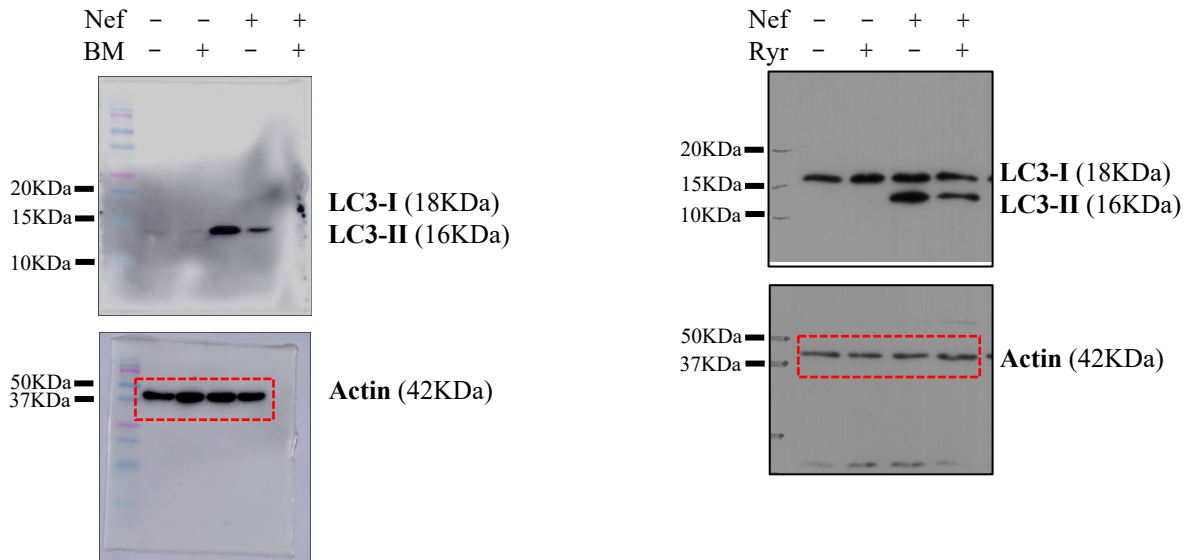
(A) Uncropped western blot images of Figure 4A. (B) Uncropped western blot images of Figure 4C.



**Uncropped western blot images of Figure 6B**



**(A)** Uncropped western blot images of Figure 7B. **(B)** Expression level of ATG7 gene in H1975 and H1650 cells. **(C)** Cell death flow cytometry analysis of neferine on Atg7 wild-type cells (H1975) and deficient cells (H1650). **(D)** The expression levels of the ATG7 gene knocked down by ATG7-siRNA in DLD-1 Bax-Bak DKO cells. **(E)** Cell death flow cytometry analysis of neferine on DLD-1 Bax-Bak DKO ATG7 wild-type cells and ATG7 knocked down cells.

HCT116 p53<sup>-/-</sup>

Uncropped western blot images of Figure 8B.